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Research Article

DEVELOPMENT AND VALIDATION OF Q-ABSORBANCE RATIO SPECTROPHOTOMETRIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF RIFAMPICIN AND ITS BIOENHANCER; 3', 5-DIHYDROXYFLAVONE-7-O-B-D-GALACTURONIDE-4'-O-B-D-GLUCOPYRANOSIDE; IN BULK AND FORMULATION

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ABSTRACT

The present research work demonstrates an analytical method development for simultaneous estimation of Rifampicin (RIF) and its bioenhancer; 3',5-dihydroxyflavone-7-O-β-D-galacturonide-4'-O-β-D-glucopyranoside (CC-I) in combined dosage form using Q-absorbance ratio concept. While method development, two different wavelengths one representing Iso-absorptive point (370 nm) and other representing the λ_{max} of Rifampicin (239 nm) were used. Optimum response was obtained in solvent system that comprises methanol and water in ratio of 80:20 v/v. Proposed UV method was found to be linear over the concentration range of 2-20 µg/ml for Rifampicin and that of 1-24 µg/ml for CC-I. On the basis of recovery studies after standard addition, accuracy of proposed method was found to be in between 99.94 to 100.30 and 99.90 to 99.96 % for RIF and CC-I respectively. Intra-day precision of the method in terms of % relative standard deviation was found to be in between 0.21 to 1.36 and 0.21 to 1.77 for RIF and CC-I respectively. Inter-day precision range of the method for RIF and CC-I was found to be in between 0.13 to 1.94 and 0.11 to 1.58 respectively. LOD and LOQ of proposed UV method were 0.043 and 0.014 µg/ml for RIF and 0.37 and 0.12 µg/ml for CC-I. Proposed UV method was robust and rugged in nature. Proposed method was successfully used for the estimation of RIF and CC-I contents of in-house formulation consisting of APIs and the common excipients.

Keywords: UV- visible spectrometry, Q absorbance ratio, Rifampicin, CC-I, Validation.

INTRODUCTION

Rifampicin (Fig.1) is a complex macrocyclic bactericidal antibiotic drug of the rifamycin group, listed in the WHO list of essential medicines [1]. It is widely used as the 1st line drug in the treatment of tuberculosis worldwide. Orally, RIF is a poorly bioavailable drug. Since long, several attempts viz. particle size reduction, solubility enhancement, formulation modification etc. [2] have been made to improvise the oral bioavailability of Rifampicin. Recently, herb-drug combinations are found to be a useful strategy to enhance the drug bioavailability [3-5]. In our earlier studies, a flavonoid glycoside (Fig.2) from *Cuminum cyminum* seeds identified as3',5-dihydroxyflavone-7-O-β-D-galacturonide-4'-O-β-D-glucopyranoside (CC-I) showed 53% enhancement in oral bioavailability of RIF [6-8]. Considering the therapeutic need of improvised and consistent oral bioavailability of Rifampicin, in-house formulation comprising RIF and CC-I was developed. For the routine analysis of the said formulation, it was envisaged that development of simple, accurate, precise yet sensitive UV-visible spectrophotometric method with ability of simultaneous estimation of both RIF as well as CC-I will be worth.

MATERIALS AND METHODS

Instrumentation

A double beam UV-visible spectrophotometer (V-530, Jasco) with spectra manager software was used for the method development and validation. Matched quartz cells with 3 cm height and 1 cm path length were used for spectral measurements. Analytical balance (Vibra HT, Essae) was used for the weighing purpose.

Material

All chemicals and reagents used for the method development purpose were of analytical or HPLC grade. Pure RIF standard was purchased from the TCI chemicals (INDIA) Pvt. ltd.

The CC-I standard was obtained as a gift sample from Natural Product Chemistry Division of Indian Institute of Integrative Medicine, Jammu & Kashmir, India.

Preparation of standard stock solution

Rifampicin and CC-I was weighed separately (5 mg each) and transferred to the 5 ml pre-calibrated volumetric flasks and dissolved in 5 ml mixture of methanol and water (80:20v/v) to

achieve a stock solution of 1000 µg/ml (Stock-1). Stock 1 was suitably diluted to achieve solution of 100µg/ml (stock 2).

Determination of maximum wavelength (λ_{max})

Stock-2 of RIF and CC-I was diluted suitably so as to obtain solutions of 10µg/ml strength. Resultant RIF and CC-I solutions were scanned over wavelength range of 800 to 200 nm using medium scanning speed. Obtained spectra were analyzed using Spectra Manager software and the λ_{max} were identified.

Preparation of calibration curve

Stock 2 of RIF was diluted suitably so as to achieve seven different calibration standards representing 2, 4, 6, 8, 10, 12 and 20 µg/ml strength whereas Stock 2 of CC-I was diluted to obtain calibration standards with 1, 2, 4, 8, 12, 16, 24 µg/ml strength. From the full spectrum measurement mode (Figure 3 and 4) of stock-2 of RIF and CC-I, two different wavelengths viz. 293 nm and 206 nm were identified as λ_{max}. The calibration curves representing concentration vs. absorbance were plotted (Figure 3 and Figure 4 respectively).

UV-spectrophotometric method

Q-Absorption ratio analysis method

Q-Absorption ratio method comprises the use of ratio of absorption at two selected wavelengths (one representing Iso-absorptive point and other representing λ_{max} of one of the two components). Proposed method is applicable to the drugs that obey Beer's law at all wavelengths and the ratio of absorbance at any two wavelengths is a constant value, independent of concentration and path length. The solutions of 14µg/ml and 24µg/ml for RIF and CC-I were scanned in the wavelength range of 400 to 200nm to obtain overlain spectra (fig 5). Two wavelengths, 370nm as Iso-absorptive point and 239nm (λmax of rifampicin) were selected for the formation of Q-absorbance ratio equation.

The concentration of the individual components was calculated by using the following equations;

$$Cx = Qm-Qy/Qx-Qy) \times A1/ax\ 1 \quad \text{(Eqn.3)}$$

$$Cy = Qm-Qy/Qy-Qx) \times A1\ /ax1 \quad \text{(Eqn.4)}$$

Where,
Qm = A2 /A1, A 1 is absorbance of sample at Iso-absorptive point,
A2 is absorbance of sample at λ_{max} of one of the two components,
Qx = ax2 /ax1, Qy = ay2 /ay1,
ax 1and ax 2 represent absorptivities of RIF at λ1 and λ2,
ay 1 and ay 2 denote absorptivities of CC-I at λ1 and λ2 respectively;
Cx and Cy be the concentration of RIF and CC-I respectively.

Validation of UV- visible spectrophotometric methods

The developed method for simultaneous estimation of RIF and CC-I was validated as per ICH guidelines. Different parameters like linearity, accuracy, precision, robustness, and ruggedness, limit of detection (LOD) and limit of quantification (LOQ) were evaluated [9-11].

Linearity and Range

Linearity of the proposed UV method was established using seven different CAL STDs of RIF and CC-I. CAL STDs of RIF and CC-I were analyzed at respective wavelengths of maximum absorbance.seven points calibration curve of RIF between the range 2-20 µg/ml and CC-I between the range 1-24 µg/ml were plotted Calibration curves in terms of absorbance vs. concentration plots were developed and subjected to linear least square regression analysis. R square value was considered to be important factor for establishing linearity of the proposed method. The interval between upper and lower concentration limit with acceptable linearity was reported to be the range of the proposed UV method.

Accuracy

Accuracy may often be expressed as % recovery by the assay of known added amount of analyte. To ascertain the accuracy of the proposed methods, recovery studies were carried at three different levels (80%, 100% and 120%) of its predefined concentration. To the predefined concentrations, different amounts of RIF and CC-I were added (standard addition method) and the accuracy was calculated on the basis of percent recovery. For calculating the percent recovery following formula was used.

$$\% RC= (SPS-S/SP) \times 100$$

Where,
SPS = Amount found in the spiked sample
S = Amount found in the sample
SP = Amount added to the sample
% RC = Percent recovery

Precision (Inter-day and Intra-day precision)

The precision of the proposed UV method was established by performing intra- and inter-day UV analysis of predefined samples. The study was performed at three concentration levels (Rifampicin: 2, 8 and 14 µg/ml and CC-I: 1,8,24 µg/ml). Samples (n=3) were analyzed at three different time intervals of a day. Study was repeated on three consecutive days. Deviation in the results was calculated in terms of % relative standard deviation (% RSD).

Robustness

Robustness of the method was assessed by analyzing MQC STDs of RIF and CC-I (8µg/ml each) at ±1nm of pre-identified wavelength of maximum absorbance for both RIF and CC-I. The results were calculated in terms of % RSD.

Ruggedness

Ruggedness of the method was established by analyzing triplicate samples of RIF and CC-I (8µg/ml each) on two different UV-Visible spectrophotometers viz. V-530, Jasco and BA-UV-2600, Bio age. Results were expressed in terms of % RSD.

Limit of Detection and Quantification

To determine the limit of detection and quantification (LOD and LOQ), the standard deviations (σ) of response and slope of calibration curve (S) were used. Detection of limit was calculated by 3.3×σ/S and quantification limit was calculated by 10×σ/S.

Table 1: Calibration data at λmax (239nm)

Sr No.	RIF		CC-I	
	Conc. (µg/ml)	Absorbance	Conc. (µg/ml)	Absorbance
1	2	0.1232	1	0.0311
2	4	0.261	2	0.0701
3	6	0.3926	4	0.1321
4	8	0.5248	8	0.2735
5	10	0.6585	12	0.4273
6	12	0.7745	16	0.5503
7	14	0.8992	24	0.8397

Table 2: Calibration data at iso-absorptive point (370nm)

Sr No.	RIF		CC-I	
	Con(µg/ml)	Absorbance	Con (µg/ml)	Absorbance
1	2	0.0821	1	0.04328
2	4	0.1705	2	0.0894
3	6	0.24583	4	0.1756
4	8	0.3529	8	0.3323
5	10	0.4056	12	0.4957
6	12	0.4865	16	0.6489
7	14	0.5684	24	0.9618

Table 3: Recovery studies for RIF and CC-I

Conc. (%)	RIF				Conc. (%)	CC-I			
	Origin level (µg/ml)	Amount Added	% Recovery	% RSD		Origin level (µg/ml)	Amount Added	% Recovery	% RSD
80	2	1.6	100.30	0.1686	80	1	0.8	99.96	1.221
100	8	8	99.94	0.1097	100	8	8	99.91	0.0571
120	14	16.8	100.25	0.4224	120	24	28.8	99.900	0.1820

Table 4: Intra-day precision data of Uv method for RIF

Sr. No			Morning			Afternoon			Evening		
	Wavelength (nm)	Conc. (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
	239		0.1251	99.15	1.220	0.1245	98.71	0.582	0.1290	102.28	0.213
1	370	2	0.0874	100.30	0.5639	0.0869	99.65	0.699	0.0878	100.72	0.347
	239		0.5244	93.24	1.0394	0.5282	93.92	0.563	0.5261	100.10	0.534
2	370	8	0.3294	100.44	1.3608	0.3275	99.86	0.466	0.3270	101.29	0.644
	239		0.8980	99.75	0.4735	0.9015	100.14	0.287	0.9018	100.18	0.277
3	370	14	0.5719	100.37	0.6908	0.5660	99.33	0.946	0.5726	100.49	1.034

Table 5: Inter-day precision data of Uv method for RIF

Sr. No	Wavelength (nm)	Conc. (µg/ml)	Day 1			Day 2			Day 3		
			Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
	239		0.1262	100.05	1.944	0.1251	99.16	0.483	0.127	100.63	1.028
1	370	1	0.0872	99.65	0.617	0.0877	100.68	0.131	0.087	100.53	0.175
	239		0.5262	100.12	0.364	0.5266	100.20	0.385	0.528	100.60	0.286
2	370	8	0.3280	100.01	0.386	0.3274	99.84	0.845	0.327	99.93	0.388
	239		0.9004	100.03	0.235	0.8983	99.79	0.260	0.900	99.99	0.202
3	370	14	0.5702	100.07	0.635	0.5720	100.39	0.930	0.571	100.21	0.801

Table 6: Intra-day precision data of Uv method for CC-I

Sr. No			Morning			Afternoon			Evening		
	Wavelength (nm)	Conc (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
	239		0.3196	101.16	1.778	0.0351	101.44	0.854	0.0368	102.12	1.391
1	370	1	0.0430	99.46	0.3546	0.0487	100.82	1.422	0.0475	98.41	1.597
	239		0.2770	100.38	1.4008	0.2825	102.36	0.743	0.2692	98.40	0.386
2	370	8	0.3374	98.55	1.3689	0.3352	100.72	5.023	0.3549	103.89	0.808
	239		0.8379	99.75	0.3277	0.8338	99.26	0.356	0.8421	100.25	0.429
3	370	24	0.9651	101.03	0.4025	0.9265	99.50	0.211	0.9763	102.21	0.361

Table 7: Inter-day precision data of Uv method for CC-I

Sr. No	Wavelength (nm)	Conc (µg/ml)	Day 1			Day 2			Day 30		
			Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	239	2	0.0364	101.59	0.162	0.0347	101.75	1.584	0.0348	102.05	0.933
	370		0.0483	99.40	1.394	0.0474	97.62	0.162	0.0482	99.17	0.838
2	239	8	0.2762	98.67	1.419	0.2776	99.15	0.113	0.2770	98.95	1.281
	370		0.3425	100.26	0.314	0.3448	100.95	0.418	0.3422	100.19	0.343
3	239	24	0.8379	99.47	0.497	0.8377	99.44	0.134	0.8368	99.34	0.433
	370		0.9560	100.08	1.413	0.957	100.18	0.198	0.9576	100.26	1.025

Table 8: Robustness study for RIF and CC-I

Conc. (µg/ml)	RIF			Conc. (µg/ml)	CC-I		
	λmax	Absorbance Mean	% RSD		λmax	Absorbance Mean	%RSD
8	239	0.5261	0.5342	8	239	0.2692	0.3865
8	240	0.5062	1.4987	8	240	0.2488	0.9034

Table 9: Ruggedness study for RIF and CC-I

Sr. No	Make of Instrument	Theoretical Conc. (µg/ml)	% RSD	
			RIF	CC-I
1	V-530, Jasco	8	0.5342	0.3865
2	UV-2600, Bio age	8	0.2113	1.6870

Table 10: LOD and LOQ for RIF and CC-I

Sr.No	Parameter	RIF	CC-I
1	LOD	0.01419	0.12353
2	LOQ	0.04302	0.37435

Table 11: Analysis of content in pharmaceutical formulation

Sr No.	Sample (n=5)	Amount present(µg/ml)	Amount found(µg/ml)	Assay%
1	RIF	8	7.97	99.62
2	CC-I	8	8.05	100.62

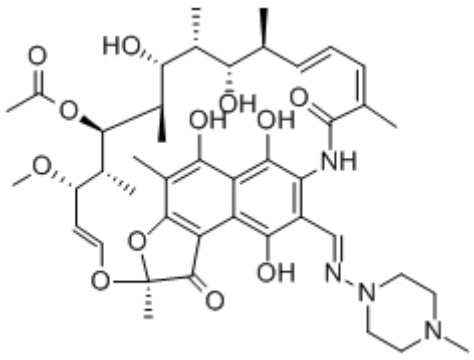

Figure 1: Chemical structure of Rifampicin

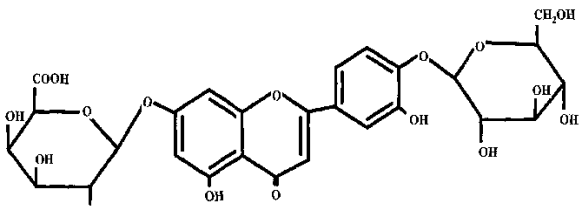

Figure 2: Chemical structure of CC-I

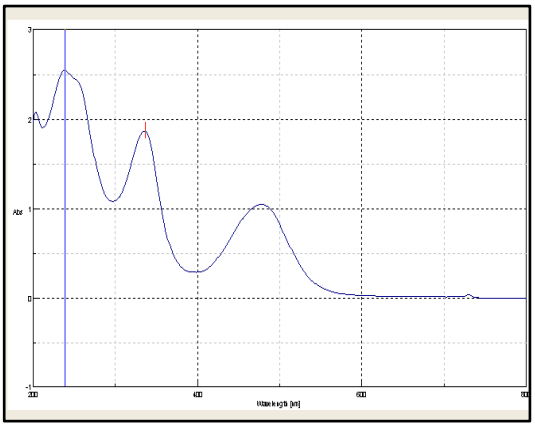

Figure 3: UV-visible spectra of RIF (239 nm)

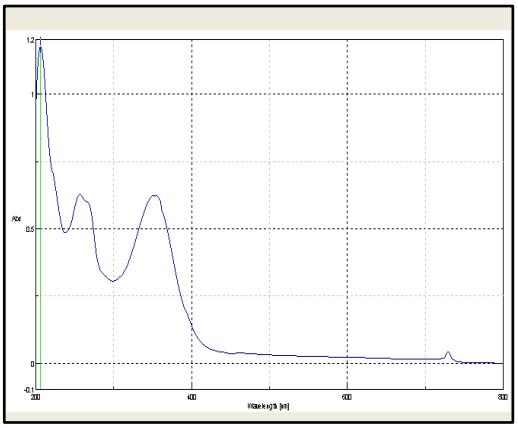

Figure 4: UV-visible spectra of CC-I (206 nm)

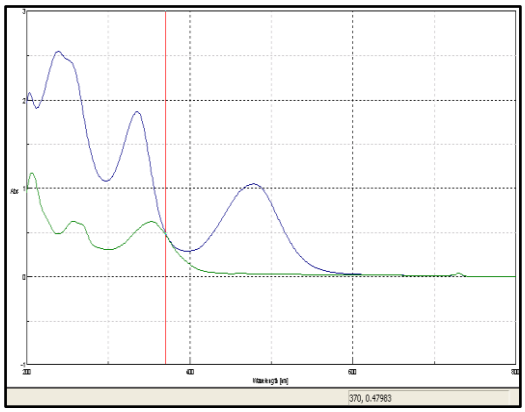

Figure 5: Overlain spectra of RIF and CC-I

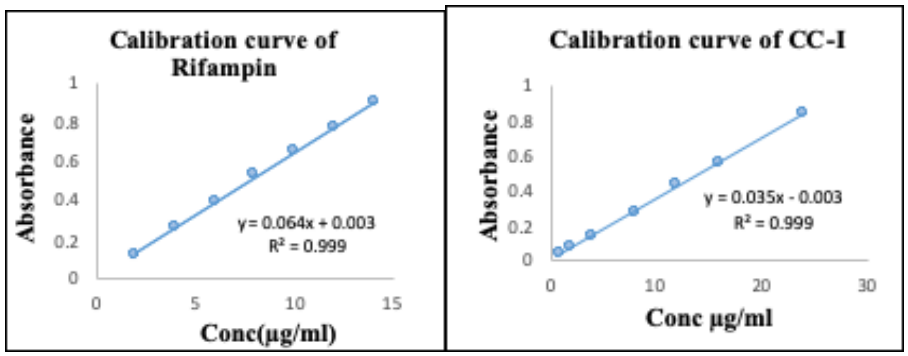

Figure 6: **Calibration curve of Rifampin and CC-I at 239 nm**

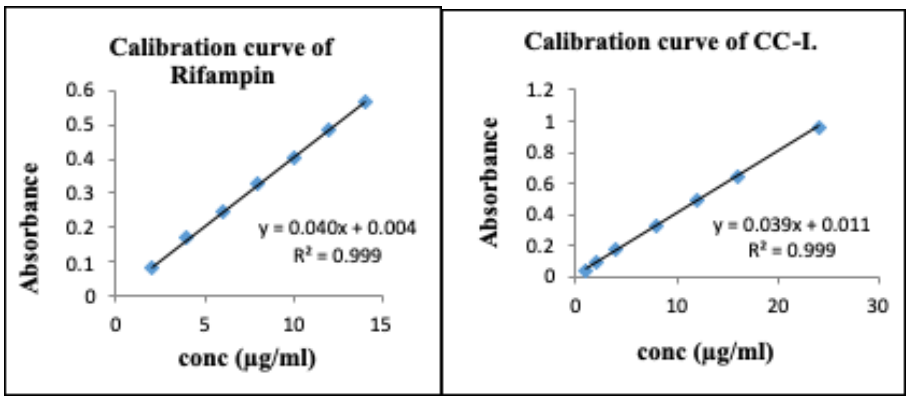

Figure 7: **Calibration curve of Rifampin and CC-I at 370 nm**

Estimation of RIF and CC-I content in pharmaceutical formulation

In-house pharmaceutical formulation of RIF and CC-I was prepared by using pharmaceutically accepted excipients viz. lactose monohydrate, talc and sodium lauryl sulfate. Briefly, 4.5 gm RIF and 0.5 gm CC-I was uniformly mixed with 4.8 gm lactose monohydrate. Obtained mixture was blended with 0.1 gm talc and sodium lauryl sulfate.

In order to estimate the contents of above-mentioned pharmaceutical formulation, 5 mg of formulation was accurately weighed and transferred to calibrated volumetric flask. The contents were dissolved in 5 ml of methanol and obtained solution was filtered through 0.45 µm syringe filter. Filtered solution was suitably diluted and analyzed for RIF and CC-I content by using proposed UV-Visible spectrophotometric method.

RESULTS AND DISCUSSION

Determination of wavelength of maximum absorbance (λ_{max})

Identification of wavelength having maximum absorbance is prerequisite for quantitative UV analysis. Solution with absorbance value less than 1 were considered to be appropriate for the determination of wavelength having maximum absorbance. The full scan of RIF and CC-I solutions resulted into identification of 477 and 284nm as the respective λmax (Fig. 3 and Fig. 4). The overlain spectra of both drugs are shown in Fig. 5.For further studies, wavelength representing Iso-absorptive point i.e. 370 nm and the λmax of RIF i.e. 239 nm were used.

Method validation

Calibration Curve for RIF and CC-I, Linearity and Range

Linearity and range are the key parameters of analytical method which demonstrates the limit within the intended method to be used for its optimum performance. Concentration and the respective mean absorbance values of RIF and CC-I are depicted in Table 1 and 2. At 239 nm, following equations were obtained for RIF and CC-I

$$y = 0.064X + 0.003 \text{ and } y = 0.035X + 0.003 \text{ (Fig 6)}$$

At 370 nm, the equations obtained for RIF and CC-I were $y = 0.040X + 0.004$ and $y = 0.039X + 0.11$ respectively (Fig. 7).
Both the calibration curves were found to be linear over the concentration range under study. Proposed UV method was found to be linear and adherence to the system of Beers Law over the concentration range of 2 to 20 µg/ml for RIF and 1 to 24 µg/ml CC-I.

Accuracy

Accuracy is the measure of how close the experimental value is to the true value. The accuracy of an analytical method expresses the closeness of agreement between the values which is accepted either as a conventional true value or an accepted reference value. Accuracy of proposed UV method for RIF and CC-I was established in terms of recovery studies. The results of the recovery studies are depicted in Table 3. Proposed method was found to be accurate

Precision

Precision of the assay was determined in terms of repeatability and intermediate precision, which was studied by comparing the assay results of 3 consecutive days. Considering the importance of reproducible and accurate results, Inter-day, intra-day precision of proposed analytical method was studied at concentrations 2, 8 and 14(µg/ml) respectively for RIF and CC-I to determine repeatability and intermediate precision.The results were expressed in terms of mean absorbance values, percent assay and % RSD for the intra-day and inter-day precision study, demonstrated in (Table 4-7) respectively for RIF and CC-I. Percent RSD values of intra-day precision study were found to be in between 0.21and 1.36 for RIF and between 0.13 and 1.94 for CC-I whereas those of inter-day precision study were found to be in between 0.21 and 1.77 for RIF and between 0.11 and 1.58 for CC-I. Percent RSD values were less than 2 demonstrated the precision of developed UV method.

Robustness

Robustness of analytical method is the ability of a method to resist the change in its performance in spite of small, deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition, buffer strength and pH etc. may occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the analytical method. Therefore, robust analytical method is preferred. The method was found to be robust as indicated by the % RSD values which are less than 2%. The % RSD values were found to be between 0.52 and 1.49 for RIF and between 0.38 and 0.90 for CC-I, shown in table 8 for RIF and CC-I respectively. Percentage RSD values were below 2 depict that the proposed UV method was robust in nature.

Ruggedness

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different instruments, different elapsed assay times, different assay temperatures and different days etc. Rugged analytical methods are free from environmental/external factors impact. For proposed UV method, sample analysis resulted into % RSD values between 0.2 and 0.5 for RIF and in between 1.6 and 0.3 for CC-I. Results showed that the proposed UV method was rugged as % RSD values were less than 2, shown in Table 9.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ represents the lowermost concentration that can be analysed with acceptable accuracy and precision. LOD and LOQ of proposed UV method were found to be 0.014 and 0.043µg/ml for RIF whereas 0.12 and 0.37 µg/ml for CC-I (Table 10). Lower LOQ values indicated that the proposed method would be sensitive enough to quantify the RIF and CC-I content of samples at its lower level.

Estimation of RIF and CC-I content in pharmaceutical formulation

The developed UV method was successfully used for estimation of RIF and CC-I content in pharmaceutical formulation. The RIF and CC-I content in the pharmaceutical formulation was found to

be 99.62 % and 100.62% respectively (Table 11) by Q-Absorbance method**.**

CONCLUSION

The simple, precise, accurate, and sensitive UV- visible spectrophotometric method for simultaneous estimation of RIF and CC-I in a bulk drug and pharmaceutical formulation was developed and validated. The recovery result confirms the high accuracy of proposed method. The proposed method was found to be robust and rugged in nature. Further, it was found that proposed method could be used for routine analysis of pharmaceutical formulation comprising RIF and CC-I.

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